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(54) Title: HEMATOLOGICAL ASSAY AND REAGENT

(57) Abstract: A hematological assay is described in which the blood coagulation potential of a body fluid is assessed by reacting a sample of the body fluid with an amount of an activator reagent comprising: (a) a predetermined amount of factor Xa or a hematologically equivalent mutant thereof, and (b) a predetermined amount of factor Va, a hematologically equivalent mutant thereof or an enzyme activating endogenous factor V, (c) (optionally) phospholipids in an aqueous solution preferably buffered to a pH from 6 to 9 (preferably 7 to 8), if desired incubating, if necessary inducing coagulation by the addition of one or more coagulation accelerants such as calcium chloride, and establishing a value indicative of the coagulation potential, e.g. by measuring the time to clotting on an optical coagulometer or through use of a chromogenic substrate. It is preferred to use at (b) factor V activator from purified Russell's Viper venom (RVV-V). An activator reagent is also described containing the components mentioned above preferably in one or more buffer solutions or in lyophilised form.

# Hematological Assay and Reagent

## Background

The present invention is concerned with the process of blood coagulation which involves an extremely complicated series of interactions generally known as the coagulation cascade, and which is well described in *Thrombosis and Hemorrhage*, Sec. Ed., 1998, published by Williams and Wilkins. It involves a series of sequential proteolytic actions directed towards coagulation and a complementary series of inhibitory actions involved with termination or inhibition of coagulation. For a better understanding of the invention Figure 1 displays a part of the blood coagulation cascade. Activation of coagulation takes place along different pathways two of which: the Intrinsic pathway and the Extrinsic pathway are illustrated. These converge to form a common pathway leading to clot formation. Coagulation factors are inactive zymogens or inactive cofactors which, when cleaved by an active protease, are activated (as indicated by the subscript "a") and in turn activate the next zymogen or the precursor of a cofactor in the cascade.

In the extrinsic pathway, damaged tissue exposes Tissue factor which activates factor VII to its activated form VIIa. Tissue factor and factor VIIIa form a complex which activates factor X at the common pathway.

In the intrinsic pathway, negatively charged surfaces are exposed to the action of factor XII and prekallikrein in the bloodstream. Factor XII is activated to factor XIIa which activates factor XI to factor XIa. Factor XIa activates factor IX to IXa. Factor IXa, Factor VIIIa, phospholipids and free calcium ions are required for the formation of the tenase complex, which activates factor X.

Factor Xa, factor Va, phospholipids and free calcium ions are required for the formation of the prothrombinase complex with which this invention is more particularly concerned, which activates prothrombin to thrombin.

Thrombin is the "key enzyme" of coagulation and is linked to many positive and negative feedback actions and also with the clotting process of blood itself. Positive feedbacks include: activation of factors V, VIII, X, and XI. Negative feedbacks include: activation of protein C in the presence of thrombomodulin. The clotting process includes the cleavage of fibrinogen to fibrin and the activation of platelets.

A number of endogenous inhibitory interactions are important, and are presented in the Figure in italics and broken lines. Thus antithrombin, an inhibitor with a relatively broad spectrum whose activity is greatly enhanced by heparins, heparinoids

and glycosaminoglycans, inhibits factor Xa, thrombin, factor XIa and factor XIIa. Heparin cofactor II is a more specific endogenous inhibitor which binds to thrombin and whose activity is also accelerated by heparin and additionally by dermatan sulfate. Activated Protein C inactivates factor VIIIa and factor Va. Tissue factor pathway inhibitor (TFPI) inhibits factor Xa and the tissue factor/factor VIIa complex in a factor Xa dependent fashion.

An appropriate equilibrium of activating and inhibiting factors is necessary for the physiological function of the coagulation system. In certain situations, e.g. hemophilia or lupus, essential factors are missing or materials (anticoagulants) are present which interfere with the coagulation system.

Also substances from animal origin can interfere with coagulation factors. For example hirudin, a protein from the salivary gland of the medicinal leech is a very potent thrombin inhibitor. Proteins from snake venoms can simulate certain factors and lead to coagulation and clotting. In particular some snake venoms simulate factor Xa and/or factor Va and can be used in *in vitro* assays for anticoagulants.

The dependence of the formation of the prothrombinase and tenase complexes on the presence of free calcium ions allows the temporary anticoagulation, e.g. with citrate (or other ionic complexing agent), of a blood sample. This allows the transport and centrifugation of a sample without clotting and also allows the performance of certain analytic reactions without the formation of said complexes.

Subsequent addition of calcium ions can immediately stimulate the activation of prothrombin to thrombin in the presence of phospholipids.

It is often necessary to treat blood with substances having a coagulating (in case of bleeding complications) or anticoagulating effect (for the prevention or treatment of thrombotic complications and during interventions which bring blood into contact with artificial materials, e.g. during extracorporeal blood circulation).

A successful long-term anticoagulation is possible with vitamin K antagonists such as warfarin or other coumarin-derived oral anticoagulants. These substances lead to impaired coagulation activity of the blood by interfering with the formation of certain coagulation factors. As they do not inhibit already formed coagulation factors they need several days, however, until a stable anticoagulation is achieved. In many situations rapid anticoagulation is necessary. One strategy for attaining anticoagulation in patients is the direct or indirect inhibition of activated coagulation factors. The inhibition of certain factors can be achieved using heparins and heparinoids, which require antithrombin and/or heparin cofactor II from plasma, and direct natural or synthetic inhibitors of factor IIa (thrombin) and Xa (e.g. hirudin, argatroban, tick anticoagulant).

All these substances mainly target activated factor X (FXa) and/or thrombin. It is important to use an appropriate intensity of anticoagulation since both too high as well as inappropriately low anticoagulation might cause a loss of organ tissue or even death of the patient.

Some anticoagulant drugs e.g. unfractionated heparin (UFH) possess highly variable pharmacokinetics and their use necessitates monitoring of the patient's condition by assaying plasma from the treated patient and, where necessary, adaptation of the individual anticoagulant dosage.

Other anticoagulant strategies such as use of the low molecular weight heparins (LMWH) do not routinely require monitoring of the anticoagulant effect, as the pharmacokinetics are usually less variable. LMWH, containing only part of the glucosaminoglycan heparin chain, is much less active than UFH and not normally used in surgery but due to its greater safety it can be prescribed for home treatment. However also with LMWH there are cases where the laboratory assessment of the drug effect is mandatory, e.g. bleeding complications under anticoagulant treatment, suspected or manifestly impaired clearance of the drug (e.g. due to renal dysfunction), unusual pharmocokinetics (e.g. in children or strongly obese patients) or suspected or potential under- and over-dosage. Prolongation of coagulation is proportional to the concentration of anticoagulant in the sample.

The European Pharmacopeial Commission has adopted a standard potency evaluation for LMWH in terms of anti-factor Xa activity (aXa).

Known coagulation assays

Methods for measuring the effect on coagulation and/or the concentration in blood or plasma of direct or indirect inhibitors of activated coagulation factors include:

(a) the assessment of inhibition of coagulation factors (e.g. FIIa and FXa) using chromogenic substrate analysis and

(b) so-called "clotting methods", e.g. the aPTT assay (activated partial thromboplastin time), the ACT assay (activated clotting time), the TT assay (thrombin time), the ECT assay (ecarin clotting time) and the Heptest® assay. The clotting methods are characterised by the fact that coagulation is activated by different regimens and the time from coagulation activation until detection of clotting in the sample is measured. The clotting time can be converted into direct concentration units by establishing a calibration curve with appropriate calibrating reagents.

Analysis of anti-factor Xa and anti-factor IIa using chromogenic substrates

Usually a plasma sample is added to a reagent containing a defined amount of factor IIa or factor Xa (in certain assays antithrombin is also added). During an incubation period factor Xa/IIa is partly inactivated by the anticoagulant itself or by complexes of the anticoagulant with endogenous or exogenous antithrombin. A chromogenic substrate is added and degraded by residual factor Xa/IIa, enhancing optical density which is detected optically. Using a calibration curve the anti-factor Xa/IIa activity or the anticoagulant concentration is calculated.

These methods allow a specific assessment of anticoagulant concentration. However, they are relatively expensive and require specialised instrumentation and are therefore not widely applied in clinical practice. Moreover, the interaction of the anticoagulant with the patient's coagulation system is not assessed and the *in vivo* situation is not directly reflected.

The aPTT assay

A blood or (more usually) a plasma sample is added to a reagent containing a contact activator (often substances with negatively charged surfaces like ellagic acid, celite or kaolin) and phospholipids, and is incubated for 2-10 minutes in the absence of calcium ions. The coagulation cascade is activated by the intrinsic coagulation pathway through the addition of calcium ions. The time recorded from the addition of $Ca^{2+}$ to the sample until detection of fibrin formation is the activated partial thromboplastin time (aPTT).

The PTT assay has the advantages that it is a widely available test, the clotting method is simple and a large experience base for the monitoring of anticoagulant therapy exists. Although the aPTT is a relatively poorly standardised method, it is frequently employed for the monitoring of unfractionated heparin (UFH), whereas LMWH cannot be monitored with this assay due to its poor responsiveness. In addition to its low sensitivity to LMWH, the assay suffers from a non-linear dose-response relationship to direct thrombin inhibitors like hirudin, too high a sensitivity to UFH (overdosage may not give a detectable endpoint), poor standardisation among different instruments, reagents, even different lots of the same reagent. Also the dose response curve for heparin is not linear.

Regarding the mechanism of the aPTT assay, it must be stated that the initiation of coagulation by contact activation (the so-called contact phase) is probably not part of the physiological hemostatic pathway in the body. The contact phase is difficult to standardise, which is one of the reasons for the very poor standardisation of the assay.

Many factors take part in the coagulation activation in the aPTT assay (XII, XI, VIII, IX, X, V, II) while inhibition of factors X and II is believed to be the main pathway of anticoagulant therapy using heparins, heparinoids and the direct inhibitors. For these reasons, the aPTT assay neither gives a very realistic estimation of the anticoagulation achieved in the patient, nor assesses anticoagulation with an appropriate specificity to the anticoagulant treatment. In addition, the assay is also sensitive to the presence of lupus anticoagulants.

The ECT assay

The Ecarin clotting time assay is a clot based assay used for monitoring the effect of direct antithrombin agents. Ecarin, a purified protease obtained from the venom of the snake *Echis carinatus*, converts prothrombin to meizothrombin (a precursor of thrombin), producing a clotting end point in citrated whole blood and plasma. Antithrombin agents such as hirudin bind to meizothrombin prolonging the Ecarin clotting time. The ECT assay is highly affected by low prothrombin levels of the sample plasma.

The ACT assay

This method consists in principle in the addition of blood to kaolin, ellagic acid or celite, and measurement of the time interval until fibrin formation in the sample. This method is widely available. It is a point-of-care method with a short turnaround time and a broad measuring range which allows monitoring of high-dose heparinisation during cardiovascular surgery.

The ACT assay has many limitations: it has a poor correlation to the anticoagulant concentration (as assessed by chromogenic substrate analysis), low sensitivity to lower heparin concentrations (up to 0.7 U UFH/ml with a normal ACT), low sensitivity to LMWH, long clotting times and a very strong dependence on the patient's coagulation factors. There is poor standardisation of different clinically applied ACT methods. The ACT can not be applied for hirudin monitoring because there is no clear relationship between the degree of prolongation and the hirudin concentration.

The thrombin time assay

The method consists in the addition of a certain amount of thrombin to the plasma sample and assessment of the time interval until clotting is detected. The method has the advantage of specifically assessing thrombin inhibition. Although this simple test is a direct measure for the antithrombin activity in plasma, it is not widely used due to its poor reliability and bad standardisation. In addition, no assessment of factor Xa

inhibition is possible. The narrow measuring range is strongly dependent on the added thrombin concentration and different results can be obtained in response to thrombin concentration, species, presence of calcium and the volume ratio of thrombin and sample.

The Heparin assay according to Yin (presented in 1973)

The assay is based in principle on the incubation of a citrated plasma sample with bovine factor Xa. After a certain incubation time, a reagent containing phospholipids and bovine plasma is added followed by a calcium chloride solution for re-calcification.

This test allows a sensitive assessment of LMWH and UFH by a clotting method. However, it involves a relatively complicated three-step procedure and requires bovine plasma (which might limit the realistic estimation of the anticoagulant effect).

Heptest® assay (variation of the Yin Assay (presented in 1987)

This assay, described in US patent 4,946,775, consists in principle in the incubation of a sample of plasma or blood with factor Xa. After a certain incubation period, a reagent containing calcium chloride, phospholipids and a bovine plasma fraction is added and the time until detection of clotting is recorded. The bovine plasma fraction is reported by the manufacturer to be rich in factor V and fibrinogen, while it is depleted of prothrombin and other coagulation factors and thus will not clot by itself.

Like the original assay of Yin, the method provides a sensitive assessment of LMWH and UFH in a relatively simple clotting assay. However the Heptest® has a low sensitivity to direct thrombin inhibitors such as hirudin; high standardisation of the bovine plasma fraction is mandatory; the effect of the bovine plasma fraction on the patient's coagulation system is not absolutely defined and its performance on optical analysers can be a problem as the optical signal is not conclusive, as will become apparent. Although the Heptest® is a simple method it is not very widely used.

The chemistry and pharmacology of heparin and assays such as those above outlined are described in *Thrombosis and Hemorrhage (op. cit.)*, and Kandrotas, R.J., *Heparin Pharmokinetics and Pharmacodynamics,* Clin. Pharmacokinet., vol. 22, 1992, pages 359 - 374.

The present invention is aimed at providing a simple and reliable hematological assay which is both sensitive and adjustable in sensitivity to cover the monitoring of a variety of anticoagulants, notably LMWH, UFH, heparinoids, dermatan sulphate, natural or synthetic inhibitors of factor Xa and inhibitors of factor IIa such as argatroban or hirudin, and in its preferred form provides a stable base line when used with optical

coagulometers. The invention has many applications in addition to the monitoring of anticoagulant treatment as will become apparent.

The following definitions are used hereafter:

Blood coagulation potential

Generally speaking the blood coagulation potential represents the ability of a patient's blood to coagulate or more specifically its ability to activate or inhibit coagulation factors. This is defined for convenience in this specification as a value, which may be given in terms of comparison with, or ratio to, a normal value or standard, of the ability of a sample, e.g. of human whole blood or plasma, or of other mammalian body fluid containing whole blood or plasma, to coagulate to the point of thrombin formation or clotting. The value may be measured in terms of the time taken from induction of coagulation e.g. by the addition to a sample of one or more coagulation accelerants such as phospholipids and calcium ions. However a value indicative of the coagulation potential can be (or can be inferred from) an indirectly measured value, e.g. an indicator value from an added analytical accessory agent, e.g. a chromogenic substrate. This may give a value e.g. for the activity of a component such as factor Xa (which activates prothrombin to thrombin) or of the activity of thrombin.

Coagulation accelerant

This is defined as a material or substance or mixture of such which greatly speeds up the rate of thrombin formation. It is preferably a substance which completes the group of substances necessary to establish the prothrombinase complex. Thus the fully assembled prothrombinase complex catalyses thrombin formation at a rate that is 300,000 times more efficient than factor Xa acting alone. In addition to factors Va and Xa the prothrombinase complex requires the presence of phospholipid (or platelets) and calcium ions (although other ions can be substituted.)

Analytical accessory agent

This is defined as an agent added to a reaction system, e.g. to a sample prior to or, more normally, following treatment to enable the provision of a conveniently observable or otherwise detectable activity. An accessory agent commonly used is a chromogenic substrate: a peptide with distinctive coloured groups which are released when the substrate is acted on by e.g. factor Xa and/or thrombin. Such agents can be specifically designed either for the detection of factor Xa activity or for the detection of thrombin formation. The use of peptide substrates is discussed in Witt, Irene, *Test*

*Systems with Synthetic Peptide Substrates in Haemostaseology*, Eur. J. Clin. Chem. Clin. Biochem., vol. 29, 1991, pages 355 - 374.

Summary of the Invention

According to a first aspect, the invention provides an activator reagent for use in blood coagulation assays comprising in combination in an aqueous solution preferably buffered to a pH from 6 to 9 (preferably7 to 8):

(a) a predetermined amount of factor Xa or a hematologically equivalent mutant thereof, and

(b) a predetermined amount of factor Va, a hematologically equivalent mutant thereof or an enzyme activating endogenous factor V,

Preferably the activator reagent also contains a predetermined amount of phospholipid.

The ingredients (a) and (b) used in the activator reagent are preferably in a purified form substantially free from other blood components such as factors and cofactors whether in activated form or not, since such impurities might affect the results obtained and/or the stability of the test system employed.

Preferably (b) consists essentially of snake venom containing factor V activator and depleted in factor X activating components. Most preferably (b) consists essentially of factor V activator from purified Russell's Viper venom (RVV-V).

The activator reagent may consist essentially of:

a predetermined amount from 0.01 to 10 nkat/ml factor Xa,

a predetermined amount from 0.05 to 5 U/ml RVV-V, and

(optionally) a predetermined amount from 1 to 200 µg/ml phospholipids,

in an aqueous buffer solution containing from 10 to 100 mM Tris/HCl, from 0.6 to 1,2% w/v NaCl and from 0.1 to 1.0% w/v albumin at a pH of from 6 to 9 (preferably 7 to 8.)

A preferred activator reagent consists essentially of

0.4 nkat/ml factor Xa,

4 U/ml RVV-V, and

50 µg/ml phospholipids from rabbit brain cephalin,

in an aqueous buffer solution containing 50 mM Tris/HCl, 0.9% w/v NaCl and 0.5% w/v albumin at a pH of 7.4.

Another preferred activator reagent consists essentially o

0.2 nkat/ml factor Xa,

2 U/ml RVV-V,

25 µg/ml phospholipids from rabbit brain cephalin, and

in an aqueous buffer solution containing 25 mM Tris/HCl, 0.45% w/v NaCl and 0.25% w/v albumin, and 12.5 mM $CaCl_2$ at pH 7.4.

The invention also includes a lyophilised preparation of an activator solution as described above.

According to a second aspect of the invention there is provided a hematological assay in which the blood coagulation potential of a body fluid is assessed by reacting a sample of the body fluid with a amount of an activator reagent comprising:

(a) a predetermined amount of factor Xa or a hematologically equivalent mutant thereof, and

(b) a predetermined amount of factor Va, a hematologically equivalent mutant thereof or an enzyme activating endogenous factor V,

in an aqueous solution preferably buffered to a pH from 6 to 9 (preferably7 to 8),

if necessary inducing coagulation by the addition of one or more coagulation accelerants, and

establishing a value indicative of the coagulation potential.

Although it is normal to employ an aqueous buffer solution as a medium for the activator reagent, the use of a buffer is not always necessary.

While the preferred assays to be described are carried out on samples of predetermined amount, e.g. weight or volume of blood or plasma, the invention is applicable in point-of-care systems which do not necessarily require an exact amount. An example of such a point-of-care system is the CoaguChek® system available from Roche Diagnostics, Mannheim.

The required value may be established by measurement of the time for a said sample of predetermined amount to a reach a (directly or indirectly) detected onset of clotting, e.g. using an optical or mechanical coagulometer, or alternatively by the addition to the treated sample of an analytical accessory agent providing a detectable value. The analytical reagent may be a synthetic substrate specific to measurement of thrombin activity or of factor Xa activity and detecting the appropriate activity. Although chromogenic substrates are better known and generally preferred, a substrate having fluorogenic, amperogenic or luminogenic properties may be utilised.

The required value may also be established by addition to the reaction system of particles exhibiting mechanical, magnetic or electrical behaviour during coagulation and detecting appropriate behaviour.

Usually the value obtained with a said sample of predetermined amount is compared with that of one or more standards or reference samples to assess the

coagulation potential. The reference sample may be a comparable sample of normal body fluid, e.g. blood or plasma or comprising blood or plasma.

The assay may be used for the determination of a blood coagulation component or treatment additive in a sample of body fluid comprising a predetermined amount of human or animal blood or plasma by comparing the coagulation potential with that of one or more standards. The coagulation potential may be compared with that of a comparable sample of normal body fluid and/or with that of a comparable sample of body fluid lacking the said component (or additive) or containing a known excess of the said component (or additive).

In a preferred assay the said value is established by detecting one of:

(i) the time from such addition to the onset of clotting,

(ii) the thrombin activity, or

(iii) the factor Xa activity.

Preferably the activator reagent includes a predetermined amount of natural or synthetic phospholipids or platelets and the method includes the addition to the reaction system of a coagulation accelerant comprising a predetermined amount of calcium (or functionally equivalent) ions.

It is preferred to utilise calcium ions as the coagulation accelerant. Phospholipids, preferably in predetermined amount, are normally added in an initial stage, e.g. in the activator reagent and/or prior to an incubation step although the invention includes the possible utilisation of phospholipids as an accelerant; in such a case calcium ions could be added with the activator reagent

The assay is preferably conducted on human plasma, but is applicable to human whole blood (or animal blood or plasma). In the preferred assay utilising calcium-bound plasma or whole blood, preferably of human origin, no prothrombinase complex is formed until coagulation is initiated by the addition of calcium ions or other coagulation accelerant. It is believed that prior to establishment or assembly of the prothrombinase complex, factor Xa, added in excess in the inventive assay, is progressively inactivated by any anticoagulant present which is effective against factor Xa. When the prothrombinase complex has been established or assembled factor Xa is protected against any further inactivation and the final activity can be determined in a stable manner. The presence of remaining factor Xa in the final optical or other determination step may contribute to the stability of the results obtained.

The assay of the invention is particularly useful for monitoring the effect upon the blood coagulation of a patient of a dosage of anticoagulant especially natural or synthetic inhibitors of factor Xa and/or thrombin (factor IIa), and in particular

unfractionated heparin (UFH), low molecular weight heparins (LMWH), dermatan sulphate, argatroban antithrombin, modified hirudin and hirudin. It may be used for monitoring the effect upon the blood coagulation of a patient of a dosage of antibody against one or more blood coagulation components or for assessing the blood coagulation potential of a patient suspected of a deficiency or superabundance of one or more blood coagulation components such as coagulation factors, which may be anticoagulant or coagulant enzymes or proenzymes.

It may be used for assessing the blood coagulation potential of whole blood or plasma suspected of the presence of an antibody against one or more blood coagulation components, e.g. lupus anticoagulant.

It is preferred to employ, as a source of the said enzyme activating endogenous factor V, a snake venom containing a factor V activating component and depleted in factor X activating component. The preferred source is factor V activator from purified Russell's Viper venom (RVV-V). This is described in e.g. Tokunaga et. al., *The Factor V-activating Enzyme (RVV-V) from Russell's Viper Venom,* Journal of Biological Chemistry, vol. 263 1988, pages 17471 - 17481 and is obtainable commercially from Pentapharm AG, Basel. Other possible venoms (suitably purified to deplete factor X activating component) include those of *Vipera lebetina*, Bothrops species, Akgistrodon species and Echis species.

A preferred method comprises the steps of:

mixing a sample of the body fluid with an amount of the activator reagent,

incubating the mixture,

adding a said accelerator, (and optionally an analytical accessory agent), and

establishing a value indicative of the coagulation potential.

In particular, the preferred assay comprises the steps of

i. preparing an activator reagent containing:

a predetermined amount from 0.01 to 10 nkat/ml factor Xa,

a predetermined amount from 0.05 to 5 U/ml RVV-V, and

(optionally) a predetermined amount from 1 to 200 µg/ml phospholipids,

in an aqueous buffer solution containing from 10 to 200 mM Tris/HCl, from 0.6 to 1.2% w/v NaCl and from 0.01 to 1.0% w/v albumin,

ii. mixing a predetermined amount from 1 to 100 µl citrated (or otherwise Ca-bound), platelet-poor plasma with a predetermined amount from 1 to 100 µl of the activator reagent,

iii. incubating the mixture,

iv. adding a predetermined amount from 10 to 100 µl of from 2 to 100 mM $CaCl_2$,

v. optionally adding an analytical accessory agent providing a detectable value, and

vi. establishing the said value.

The buffer is preferably at a pH from 6 to 9, more preferably 7 to 8 and most preferably pH 7.4

More preferably the method comprises the steps of

i. preparing an activator reagent containing:

0.4 nkat/ml factor Xa,

4 U/ml RVV-V, and

50 µg/ml phospholipids from rabbit brain cephalin,

in an aqueous buffer solution containing 50 mM Tris/HCl, 0.9% w/v NaCl and 0.5% w/v albumin at pH 7.4,

ii. mixing 50 µl citrated platelet-poor plasma with 50 µl of the activator reagent,

iii. incubating the mixture for 3 minutes at 37°C,

iv. adding 50 µl of from 25 mM $CaCl_2$, and

v. optionally adding an analytical accessory agent providing a detectable value, and

vi. establishing the said value.

While the incubation step is normally used, in a modification of the assay described above useful for assessing high concentrations of heparin and similar anticoagulants the assay may be carried out in the absence of incubation. A preferred assay of this kind comprises the steps of

i. preparing an activator reagent containing:

0.2 nkat/ml factor Xa,

2 U/ml RVV-V,

25 µg/ml phospholipids from rabbit brain cephalin,

in an aqueous buffer solution containing 25 mM Tris/HCl, 0.45% w/v NaCl and 0.25% w/v albumin, and 12.5 mM $CaCl_2$, at pH 7.4

ii. mixing 50 µl citrated, platelet-poor plasma with 100 µl of the activator reagent,

iii. optionally adding an analytical accessory agent providing a detectable value, and

iv. establishing the said value.

Preferably the said value is established by measuring the time from addition of $CaCl_2$ to the onset of clotting using an optical coagulometer.

In an assay employed for assessing the presence and/or concentration of a suspected coagulant or anticoagulant component in a plasma sample the sample may be

diluted in plasma depleted only in the suspected coagulant or anticoagulant and the value established compared with that found with the depleted plasma and/or normal plasma.

A plasma sample may also be pre-diluted with normal plasma, a plasma fraction or one or more single coagulation factors so as to reduce the influence of matrix effects or to minimize the dependency of plasma coagulation factors, or it may be treated with a substance inactivating heparin or heparin-like substances thereby enhancing specificity to non-heparin anticoagulants. The assay may be carried out on a plasma sample which has been treated with a substance which activates or inactivates one or more coagulation factors.

The invention includes a kit for use in blood coagulation assays comprising in combination:

(i) an activator reagent as described above or separate components thereof,

(ii) control and/or calibration samples, and

(iii) one or more optional accessories selected from

an aqueous solution of a calcium (or equivalent) salt of stated concentration, an analytical accessory agent, normal plasma, one or more plasma fractions, one or more coagulation factors, water and/or one or more buffer solutions. The kit will include instructions for carrying out an assay as described. Equivalent lyophilised preparations can be used.

The analytical accessory agent may comprise one or more solutions or lyophilised preparations of synthetic substrates for thrombin and/or factor Xa determination.

Brief description of the drawings

Preferred forms of the invention are hereafter described with reference to the accompanying drawings, in which:-

Figure 1 is a diagram (described above) illustrating the blood coagulation cascade,

Figure 2 is a graph illustrating typical dose response lines for LMWH, UFH and hirudin in a Heptest®assay,

Figure 3 is a graph showing optical signal curves produced in a typical Heptest® assay,

Figure 4 is a diagram illustrating the principle of the present assay,

Figure 5 is a graph similar to Figure 2 illustrating typical dose response lines for LMWH, UFH and hirudin in an assay according to the invention,

Figure 6 is a graph similar to Figure 3 showing optical signal curves produced in an assay according to the invention,

Figure 7 is a graph illustrating the effect produced by adding additional amounts of factor Xa,

Figure 8 is a graph illustrating the effect produced by omitting the incubation step,

Figure 9 is a graph illustrating results obtained using the inventive assay and using the Heptest® on a patient treated with an oral anticoagulant, and.

Figure 10 is a graph showing the correlation of assays according to the invention with assays performed according to the antifactor Xa assay, performed on volunteers treated with LMWH.

Preferred forms of the invention

For comparison with the assay according to the invention, Figure 2 shows the dose-response relationship of the Heptest® with the common anticoagulants LMWH, UFH and recombinant hirudin (r-hirudin) in human normal reference plasma . This figure demonstrates the relatively poor responsiveness of the Heptest® for these direct thrombin inhibitors, in this case of hirudin.

Figure 3 shows optical signals of the Heptest® assay on the Behring Coagulation System (BCS) microcoagulometer at 405 nm. The baseline of the combination sample (citrated plasma prepared as recommended in the Heptest® package) is not stable. A decrease of absorbency does not allow the exact detection of the beginning of clot formation. The entire reaction curve displays noise which may have a negative influence on precision, a disadvantage when modern automated coagulometers are used for endpoint detection.

A preferred assay according to the invention permits the assessment of the coagulation potential of samples using a clotting method, based on the use of two, and preferably three, activating agents in predetermined concentrations together with the use of calcium ions, normally to initiate the beginning of clotting. Although this method, like the Yin assay and the Heptest® assay relies upon the formation of a prothrombinase complex on phospholipids, many or most of the disadvantages mentioned, including poor optical signals as described with reference to Figure 3, are overcome. This new method is sensitive for UFH , LMWH and hirudin and other direct or indirect factor Xa and/or thrombin inhibitors in the relevant concentration ranges and in clinical practice. Moreover, it provides very stable optical signals.

In the preferred method of performing the assay, a blood or plasma sample (from which calcium ions are absent or have been complexed or bound e.g. by citration) is incubated with a defined amount of factor Xa, phospholipids and factor V-activating enzyme, preferably using a specially prepared activator reagent. After an incubation period the sample/reagent mixture is re-calcified, e.g. with $CaCl_2$ and the time to the onset of clotting (clotting time) is observed and recorded. By comparison with Figure 1,

Figure 4 illustrates the activation procedure diagrammatically. Components of the activator reagent are shown in large bold letters. Activation is believed to rely on the establishment of a prothrombinase complex, consisting of a predetermined amount of factor Xa, patient-own factor V activated using a predetermined amount of factor V-activator from Russell's Viper venom (RVV-V), phospholipids and $CaCl_2$. Re-calcification of citrated blood or plasma is employed to complete the assembly of the prothrombinase complex on the phospholipid surface and clotting begins.

By contrast to previous methods such as the Yin assay in which factor Va is generated by positive feedback activation when thrombin is formed, the inventive method employs a thrombin independent step for immediate complete activation of factor V of the sample to Va. In a modification, excess factor V and/or other factors can be added in order to make the test system independent from changes in the activity of this factor. The added factors can be simply plasma or a plasma fraction. Purified or recombinant factors can be used or suitable mutations.

Example 1

Preparation of the activation reagent

An activator reagent was prepared having the following composition:

| | |
|---|---|
| NaCl in aqueous solution | 0.9% w/v |
| Tris/HCl buffer, pH 7.4[1] | 50 mM |
| albumin[2] | 0.5% w/v |
| factor Xa (FXa)[3] | 0.4 nkat/ml |
| Russell's Viper venom factor V activator (RVV-V)[4] | 4 U/ml |
| phospholipids[5] | 50 µg/ml |

Assay procedure

50 µl citrated, platelet-poor plasma were mixed with 50 µl of the activator reagent and various amounts of the anti-clotting agents LMWH[6], UFH[7] and r-hirudin[8] to form test samples. The samples were incubated for 180 seconds at 37°C. 50 µl of 25 mM $CaCl_2$ solution were then added to each sample and the time to clotting measured on a BCS (Dade-Behring) optical coagulometer. The results in terms of anti-coagulant concentration are shown in Figure 5.

Comparison of Figure 5 with Figure 2 shows a great improvement in sensitivity (approaching a factor of 2) over the Heptest®. By contrast to the Heptest® the inventive assay is also very sensitive for hirudin.

The optical signals for the assay are shown in Figure 6. A very stable baseline is exhibited at about 0.5 nm on the Behring Coagulation System by comparison with Figure 3 and there is a sharp increase in turbidity at the onset of clotting. The signals are very conclusive and display a low noise level. It can therefore be expected that the assay can easily be adapted to different instruments with optical or mechanical detection of the onset of coagulation.

1 from Sigma, Munich

2 human serum albumin from Centeon, Marburg

3 FXa supplied by Chromogenix, Essen

4 from Pentapharm AG, Basel

5 rabbit brain cephalin from Pentapharm AG, Basel

6 WHO standard LMWH from Chromogenix, Essen

7 unfractionated heparin from B. Braun-Melsungen, Melsungen

8 recombinant hirudin (Lipirudin® from Hoechst Marrion Roussel, Bad Soden

Reference plasma was obtained from Immuno, Vienna and reconstituted as instructed.

Example 2

Variation of factor Xa concentration

It was unexpectedly found that by varying the added factor Xa concentration, the sensitivity of the activation procedure could be adjusted over a wide range.

Procedure

The procedure of Example 1 was followed with the exception that activating reagents with different concentrations of factor Xa (FXa) from 0.1 to 1.6 nkat/ml were prepared and test samples prepared with 0, 0.5 and 1 U/ml of LMWH. The results are shown in Figure 7. It can be seen that unexpectedly good signals were obtained even with reduced concentration of factor Xa and a stable base line achieved even with heparinised samples.

This discovery enables assays to be designed for a broad spectrum of unknown anti-coagulant concentrations, and a variety of different applications are possible, including:

a) Assays for factor Xa and thrombin inactivation by the assessment of thrombin activity using substrates (e.g. chromogenic substrates) which give detectable signals for thrombin.

b) Assays for factor V inactivation by added activated protein C or endogenous protein C activated using appropriate enzymes (e.g. Protac® or thrombin/thrombomodulin).

c) Assays of other unknowns such as activators, inhibitors or substrates which give detectable signals with factor Xa or thrombin.

Example 3

Variation of the incubation period

It was also found that performing the activation procedure without an incubation step leads to a very extended measuring range, especially for unfractionated heparin, which is useful in assessing high anticoagulant concentrations.

Preparation of the activation reagent

The activator reagent of Example 1 was diluted with an equal volume of 0.025 M calcium chloride solution to produce a reagent having the following composition:

| | |
|---|---|
| NaCl in aqueous solution | 0.45% w/v |
| Tris/HCl buffer | 25 mM (pH 7.4) |
| albumin | 0.25% w/v |
| factor Xa (FXa) | 0.2 nkat/ml |
| Russell's Viper venom factor V activator (RVV-V) | 2 U/ml |
| phospholipids | 25 µg/ml |
| $CaCl_2$ | 12.5mM |

Procedure

50 µl test samples of citrated platelet-poor plasma containing varying amounts of LMWH, UFH and r-hirudin were mixed with 100 µl activator reagent and the time to onset of clotting measured as in Example 1. The results shown in Figure 8 demonstrate a broad measuring range with particularly good results for UFH.

Example 4

Patient on oral anticoagulant treatment

Analysis of samples from patients with reduced factor activity following treatment with additional, e.g. oral anticoagulants showed a much lower apparent deficiency in the new assay than in the Heptest® assay. This may be due to the strong direct factor V activation in the new procedure which minimises a potential delay of thrombin mediated factor V activation when thrombin formation is itself retarded due to a reduced factor activity. Thus anticoagulant concentrations can be assessed more reliably using the new assay in such circumstances.

In this example samples were prepared from blood from a patient treated with an oral anticoagulant with an International Normalised Ratio (INR) of 3,2. Presently used oral anticoagulants are coumarin derivatives such as warfarin. These are competitive inhibitors of vitamin K in the gamma-carboxylation of Vitamin K dependent coagulation factors and primarily the respective carboxyproteins lacking in calcium binding capacity are formed. The activity of these factors is reduced.

The samples contained added amounts of 0, 0.25 and 0.5 aXa U LMWH. These samples were subjected to a Heptest® and to the procedure of Example 1. The results are shown graphically in Figure 9. It will be seen that the oral anticoagulant treatment had little or no effect on the results for LMWH activity in the inventive assay whereas a sharp difference appeared in the Heptest®.

Example 5

Use of a chromogenic substrate

The following procedure may be used.

50 µl samples of citrate, fresh frozen plasma containing quantities of LMWH, UFH and r-hirudin (as in Example 1) are incubated with 87.4 µl of activator reagent (Example 1) for 5 minutes at 37°C. An accelerant solution is then added containing:

| | |
|---|---|
| Tris/HCl buffer, 50 mM in 0.9% w/v NaCl, pH 7.4 | 562.5 µl |
| Pefabloc(r), 10 mg/ml in 0.9% w/v NaCl | 100 µl |

$CaCl_2$, 25 mM 100µl

Chromogenic substrate for thrombin[1], 4 mM in water 100 µl

1 e.g. Tos-Gly-Pro-Arg-pNaAcOH from Pentapharm AG..

The solutions are mixed and the change in optical density measured at 405 nm after incubation for 5 minutes at 37°C.

Example 6

Comparison of the inventive assay with an anti-factor Xa assay.

The inventive assay was compared to an anti-factor Xa activity assay using a chromogenic substrate. 30 samples taken from volunteers under LMWH treatment were assayed following the procedure of in Example 1 and using the "antifactor Xa activity assay" from Chromogenix, Mölndal, Sweden, following makers instructions. The results, as plotted in Figure 10, demonstrate a good correlation.

Variations

The present activator reagent and activation procedure gives rise to numerous variations in assays and in other applications. In particular the procedure may be varied to assay an unknown sample of one of the ingredients described. Some variations are illustrated below.

1. Variations involving the detection of the onset of coagulation

i) Thrombin formation may be detected by the addition of a synthetic substrate known for this purpose. The substrate may have chromogenic, fluorogenic, amperogenic, luminogenic or other measurable property. The substrate may for example be cleaved by thrombin to leave a detectable group.

ii) Known detection methods may be used for measuring viscosity, elasticity, flow characteristics, clot resonance, the movement of erythrocytes or the behaviour of added objects such as particles which alter their behaviour upon the onset of coagulation. For example the oscillation of added magnetic particles may be measured or the mechanical characteristics of added particles.

iii) Rapid immunological detection procedures with thrombin specific antibodies may be applied, e.g. in conjunction with plasmon resonance or similar techniques.

2. Variations involving factor Xa

Instead of adding a defined amount of factor Xa from bovine, human or other origin to the sample a substance which activates endogenous factor Xa could be used, or a substance with a similar function to factor Xa, e.g. mutants of factor Xa, snake venom enzymes, or factor Xa-activating cysteine proteinase from tumor cells.

3. Variations involving factor V

Instead of activating endogenous factor V in plasma or other source by the addition of RVV-V or factor V activators from other snake venoms such as Akgistrodon species, Bothrops species, *Vipera lebetina*, Echis species or alternative activating substance, certain applications may employ already activated factor V or a substance with a similar function to factor V, e.g. a mutant of factor V.

4. Variations involving phospholipids

Certain applications may employ other sources of phospholipids, e.g.:

i) The contribution of the patient's own phospholipid structures to coagulation may be assessed, e.g. by using a sample in which platelets are present.

ii) Phospholipids may be assayed by using a two step procedure in which in one of the steps the unknown phospholipid is added.

iii) The concentration of phospholipids can be adjusted in order to minimise where necessary the potential interference of lupus anticoagulants or anti-phospholipid antibodies.

The phospholipids may be of human, animal, plant or synthetic origin or a mixture of such.

5. Variations involving anticoagulants

The assay procedure can be made specific to heparin-independent inhibitors of factor X, II or V by the addition of substances which inactivate heparin, e.g. protamine, heparinase or polybren.

6. Variations involving plasma

The procedure may be performed with the addition of plasma, plasma fractions or single factors in order to correct factor deficiencies or in order to make the test more susceptible or less susceptible to certain mechanisms. Such added substance may include e.g.:

i) Human normal plasma or plasma fractions or single factors or similar mutants.

ii) Bovine (or other animal) normal plasma or plasma fractions or single factors or similar mutants.

7. Variations involving other factors

The procedure may be performed with the addition of synthetic substrates, activators or inhibitors of any one or selected combination of factors II, IIa, V, Va, X and Xa.

Conclusion

In conclusion, the present invention includes a simple plasma clotting assay based on defined, readily available and easy to stabilise components. The results show linear dose-response relationships for LMWH, hirudin and UFH. These very good dose-response relationships appear to rely on anti-factor IIa and anti-factor Xa activity which is independent of earlier stages of the coagulation cascade and independent of factor V activation during the initiation of coagulation by thrombin generated from the samples' coagulation factors.

In contrast to techniques which activate prothrombin by prothrombin activators from snake venoms (e.g. the ECT method for hirudin determination), the present technique uses the physiological pathway of prothrombin activation (using factor Xa, factor Va, phospholipids and calcium ions), which is of advantage for the assessment of physiologically relevant coagulation mechanisms and processes.

The good optical signal, high precision, short measuring times and the ease of performance using the standard procedures of the aPTT and PT allow adaptability of the new procedure to different coagulation analysers.

Comparison of the results of the new procedure, based on preformed prothrombinase, and the Heptest® which employs factor Xa only, prior to incubation, shows that the two tests present significant differences. The Heptest® had longer

coagulation times with the LMWH samples when compared to the inventive assay, and its sensitivity was slightly lower for UFH and dramatically lower for hirudin. Compared to the Heptest® the new method works on a defined physiological basis, does not require bovine plasma fraction, activates factor V independently from thrombin, has an improved optical signal and shows high sensitivity to hirudin. Therefore it can be used to monitor not only UFH and LMWH but also hirudin with the same reagent in relevant concentration ranges.

By contrast to the invention, the Russell's Viper venom time assay employs both factor V and factor X activation by the venom, which results in varying factor Xa concentrations depending on the patient's factor X concentration. A more defined activation is achieved in the invention by the application of a defined excess factor Xa activity.

Finally, the invention provides a procedure which allows a simple assay for the monitoring of LMWH, heparinoids, hirudins and UFH based on a single principle. A variety of further tests are possible when the activation regimen is combined with substances which activate or inhibit processes which affect factor V, X or II directly or indirectly (like the protein C system), or the use of indicator substrates.

CLAIMS

1. An activator reagent for use in blood coagulation assays comprising in combination in an aqueous solution preferably buffered to a pH from 6 to 9 (preferably7 to 8):

(a) a predetermined amount of factor Xa or a hematologically equivalent mutant thereof, and

(b) a predetermined amount of factor Va, a hematologically equivalent mutant thereof or an enzyme activating endogenous factor V,

2. An activator reagent according to claim 1 also containing a predetermined amount of phospholipid.

3. An activator reagent according to claim 1 or claim 2 wherein (b) consists essentially of snake venom containing factor V activator and depleted in factor X activating components.

4. An activator reagent according to claim 3 wherein (b) consists essentially of factor V activator from purified Russell's Viper venom (RVV-V).

5. An activator reagent according to claim 4 consisting essentially of

a predetermined amount from 0.01 to 10 nkat/ml factor Xa,

a predetermined amount from 0.05 to 5 U/ml RVV-V, and

(optionally) a predetermined amount from 1 to 200 µg/ml phospholipids,

in an aqueous buffer solution containing from 10 to 100 mM Tris/HCl, from 0.6 to 1.2% w/v NaCl and from 0.1 to 1.0% w/v albumin at a pH of from 6 to 9 (preferably 7 to 8).

6. An activator reagent according to claim 4 consisting essentially of

0.4 nkat/ml factor Xa,

4 U/ml RVV-V, and

50 µg/ml phospholipids from rabbit brain cephalin,

in an aqueous buffer solution containing 50 mM Tris/HCl, 0.9% w/v NaCl and 0.5% w/v albumin at a pH of 7.4.

7. An activator reagent according to claim 4 consisting essentially of

0.2 nkat/ml factor Xa,

2 U/ml RVV-V, and

25 µg/ml phospholipids from rabbit brain cephalin,

in an aqueous buffer solution containing 25 mM Tris/HCl, 0.45% w/v NaCl and 0.25% w/v albumin, and 12.5 mM $CaCl_2$ at pH 7.4.

8. A lyophilised preparation of an activator solution according to any preceding claim.

9. A hematological assay in which the blood coagulation potential of a body fluid is assessed by reacting a sample of the body fluid with an activator reagent comprising:

(a) a predetermined amount of factor Xa or a hematologically equivalent mutant thereof, and

(b) a predetermined amount of factor Va, a hematologically equivalent mutant thereof or an enzyme activating endogenous factor V,

in an aqueous solution preferably buffered to a pH from 6 to 9 (preferably7 to 8),

if necessary inducing coagulation by the addition of one or more coagulation accelerants, and establishing a value indicative of the coagulation potential.

10. An assay according to claim 9 wherein a snake venom containing a factor V activating component and depleted in factor X activating component is employed as a source of the said enzyme activating endogenous factor V.

11. An assay according to claim 10 wherein the said source is factor V activator from purified Russell's Viper venom (RVV-V).

12. An assay according to any of claims 9 to 11 in which the coagulation potential of a said sample of predetermined amount is compared with that of one or more standards.

13. An assay according to any of claims 9 to 12 in which the establishment of said value comprises detecting one of:

(i) the time from such addition to the onset of clotting,

(ii) the thrombin activity,

(iii) the factor Xa activity.

14. An assay according to any of claims 9 to 13 wherein the activator reagent includes a predetermined amount of natural or synthetic phospholipids or platelets and the method includes the addition to the reaction system of a coagulation accelerant comprising a predetermined amount of calcium (or functionally equivalent) ions.

15. An assay according to any of claims 9 to 14 wherein the said value is established by measurement of the time for a said sample of predetermined amount to a reach a detected onset of clotting using an optical coagulometer.

16. An assay according to any of claims 9 to 14 wherein the said value is established by the addition to the treated sample of an analytical accessory agent providing a detectable value.

17. An assay according to claim 16 wherein the said value is established by addition to the reaction system as an analytical accessory agent of a synthetic substrate specific to measurement of thrombin activity or specific to measurement of factor Xa activity and detecting the appropriate activity.

18. An assay according claim 17 wherein the said substrate has chromogenic, fluorogenic, amperogenic or luminogenic properties.

19. An assay according to claim 16 wherein the said value is established by addition to the reaction system as an analytical accessory agent of particles exhibiting mechanical, magnetic or electrical behaviour during coagulation and detecting appropriate behaviour.

20. An assay according to any of claims 9 to 19 in which the determination of a blood coagulation component or treatment additive in a sample of body fluid comprising a predetermined amount of human blood or plasma is performed by comparing the said coagulation potential with that of one or more standards.

21. An assay according to any of claims 9 to 19 in which the determination of a blood coagulation component or treatment additive in sample of body fluid comprising a predetermined amount of human blood or plasma is performed by comparing the said coagulation potential with that of a comparable sample of normal body fluid and/or with that of a comparable sample of body fluid lacking the said component (or additive) or containing a known excess of the said component (or additive).

22. An assay according to any of claims 9 to 21 employed for monitoring the effect upon the blood coagulation of a patient of a dosage of anticoagulant.

23. An assay according to claim 22 wherein the anticoagulant is selected from natural or synthetic inhibitors of factor Xa and/or thrombin.

24. An assay according to claim 22 or claim 23 wherein the anticoagulant is selected from unfractionated heparin (UFH), low molecular weight heparins (LMWH), heparinoids, dermatan sulphate, antithrombin, argatroban, modified hirudin and hirudin.

25. An assay according to any of claims 9 to 24 employed for monitoring the effect upon the blood coagulation of a patient of a dosage of antibody against one or more blood coagulation components.

26. An assay according to any of claims 9 to 25 employed for assessing the blood coagulation potential of a patient suspected of a deficiency or superabundance of one or more blood coagulation components.

27. An assay according to claim 26 wherein the said component is a coagulation factor or an anticoagulant enzyme or proenzyme.

28. An assay according to any of claims 9 to 27 employed for assessing the blood coagulation potential of whole blood or plasma suspected of the presence of an antibody against one or more blood coagulation components.

29. An assay according to claim 28 employed for assessing the presence in a sample of whole blood or plasma of lupus anticoagulant.

30. An assay according to any of claims 9 to 29 wherein the method comprises the steps of:

mixing a sample of the body fluid with an amount of the activator reagent,

incubating the mixture,

adding a said accelerator, (and optionally an analytical accessory agent), and

establishing a value indicative of the coagulation potential.

31. An assay according to claim 30 comprising the steps of

i. preparing an activator reagent containing:

a predetermined amount from 0.01 to 10 nkat/ml factor Xa,

a predetermined amount from 0.05 to 5 U/ml RVV-V, and

(optionally) a predetermined amount from 1 to 200 µg/ml phospholipids,

in an aqueous buffer solution containing from 10 to 200 mM Tris/HCl, from 0.6 to 1.2% w/v NaCl and from 0.01 to 1.0% w/v albumin, pH 6 to 9 (preferably 7 to 8)

ii. mixing a predetermined amount from 1 to 100 µl citrated (or otherwise Ca-bound), platelet-poor plasma with a predetermined amount from 1 to 100 µl of the activator reagent,

iii. incubating the mixture,

iv. adding a predetermined amount from 10 to 100 µl of from 2 to 100 mM $CaCl_2$,

v. optionally adding an analytical accessory agent providing a detectable value, and

vi. establishing the said value.

32. An assay according to claim 31 comprising the steps of

i. preparing an activator reagent containing:

0.4 nkat/ml factor Xa,

4 U/ml RVV-V, and

50 µg/ml phospholipids from rabbit brain cephalin,

in an aqueous buffer solution containing 50 mM Tris/HCl, 0.9% w/v NaCl and 0.5% w/v albumin at pH 7.4;

ii. mixing 50 µl citrated platelet-poor plasma with 50 µl of the activator reagent,

iii. incubating the mixture for 3 minutes at 37°C,

iv. adding 50 µl of from 25 mM $CaCl_2$, and

v. optionally adding an analytical accessory agent providing a detectable value, and

vi. establishing the said value.

33. A modification of the assay claimed in claim 31 or claim 32 useful for assessing high anticoagulant concentrations in which the assay is carried out in the absence of incubation.

34. An assay according to claim 33 comprising the steps of

i. preparing an activator reagent containing:

0.2 nkat/ml factor Xa,

2 U/ml RVV-V,

25 µg/ml phospholipids from rabbit brain cephalin,

in an aqueous buffer solution containing 25 mM Tris/HCl, 0.45% w/v NaCl and 0.25% w/v albumin, and 12.5 mM $CaCl_2$, at pH 7.4

ii. mixing 50 µl citrated, platelet-poor plasma with 100 µl of the activator reagent,

iii. optionally adding an analytical accessory agent providing a detectable value, and

iv. establishing the said value.

35. An assay according to any of claims 31 to 34 wherein the said value is established by measuring the time from addition of $CaCl_2$ to the onset of clotting using an optical coagulometer.

36. An assay according to any of claims 9 to 35 employed for assessing the presence and/or concentration of a suspected coagulant or anticoagulant component in a plasma or whole blood sample in which the sample is diluted in plasma depleted only in the suspected coagulant or anticoagulant and the value established compared with that found with the depleted plasma and/or normal plasma.

37. An assay according to any of claims 9 to 36 in which a plasma sample is diluted with normal plasma, a plasma fraction or one or more single coagulation factors so as to reduce the influence of matrix effects.

38. An assay according to any of claims 9 to 37 in which a plasma sample is treated with a substance inactivating heparin or heparin-like substances thereby enhancing specificity to non-heparin anticoagulants.

39. An assay according to any of claims 9 to 38 carried out on a plasma sample which has been treated with a substance which activates or inactivates one or more coagulation factors.

40. An assay according to claim 9 carried out on a point-of-care system.

41. A kit for use in blood coagulation assays comprising in combination:

(i) an activator reagent according to any of claims 1 to 8 or separate components thereof,

(ii) control and/or calibration samples, and

(iii) one or more optional accessories selected from

an aqueous solution of a calcium (or equivalent) salt of stated concentration, an analytical accessory agent, normal plasma, one or more plasma fractions, one or more coagulation factors, water and/or one or more buffer solutions,

or equivalent lyophilised preparations,

together with instructions for carrying out an assay according to any one of claims 9 to 40.

42. A kit according to claim 41 wherein the analytical accessory agent comprises one or more solutions or lyophilised preparations of synthetic substrates for thrombin and/or factor Xa determination.

Fig. 1

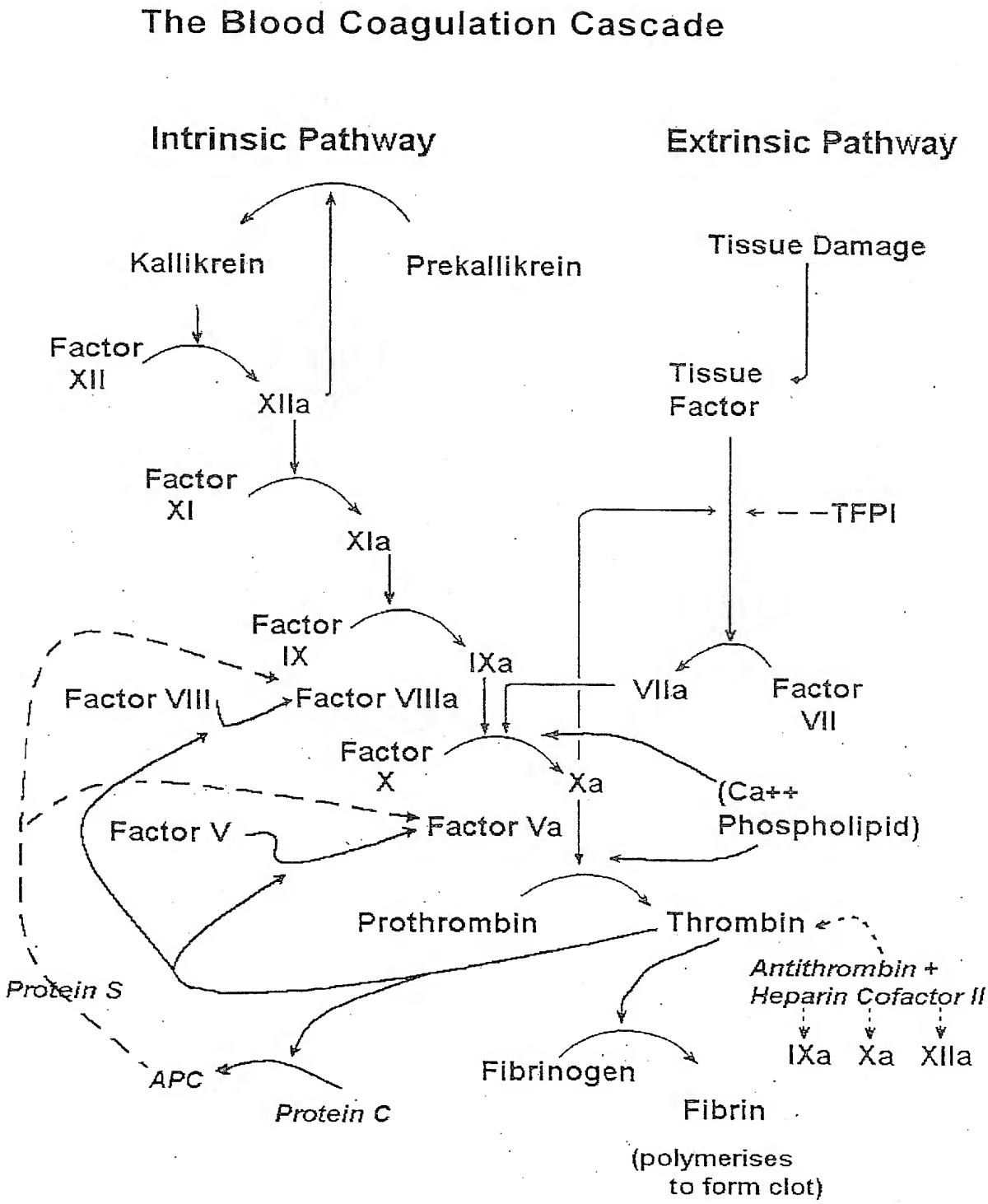


Fig. 2

Heptest® Dose Response

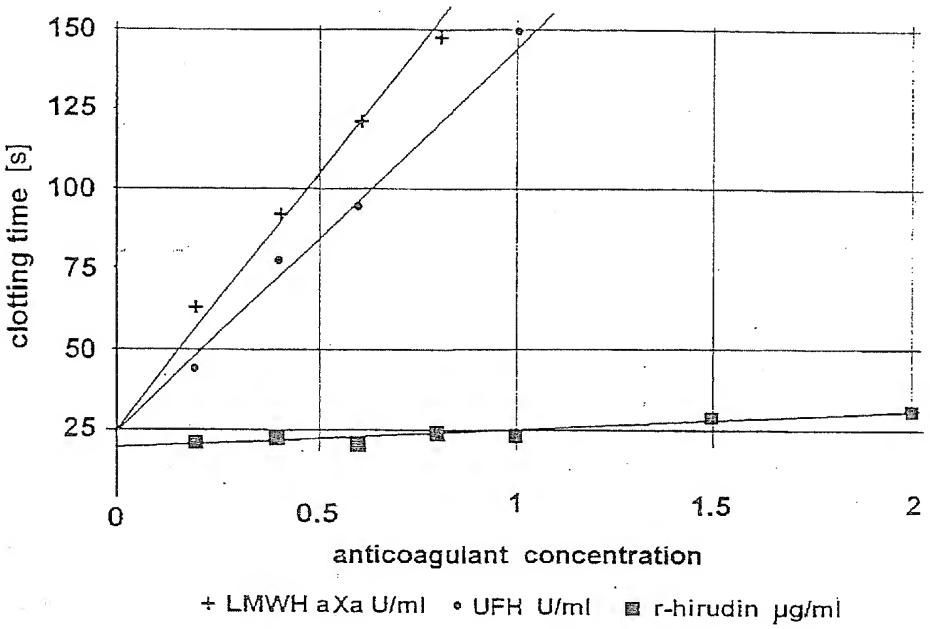
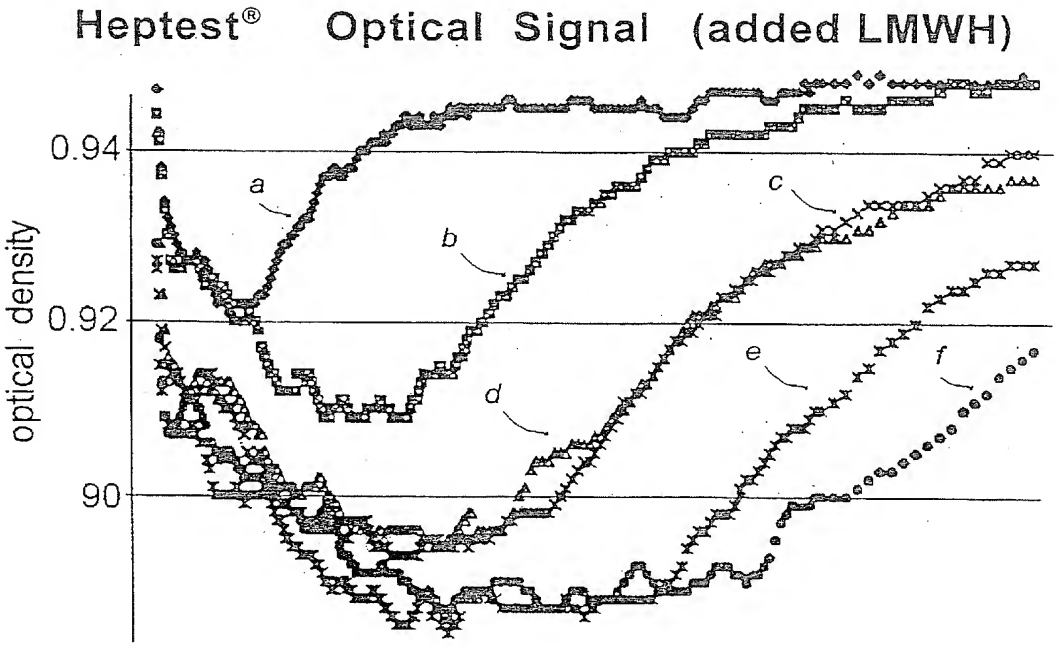



Fig. 3

Heptest® Optical Signal (added LMWH)




*a control, b 0.2 aXa U/ml, c 0.4 aXa U/ml, d 0.6 aXa U/ml, e 0.8 aXa U/ml, f 1 aXa U/ml*

Fig. 4

**Principle of the new clotting assay**

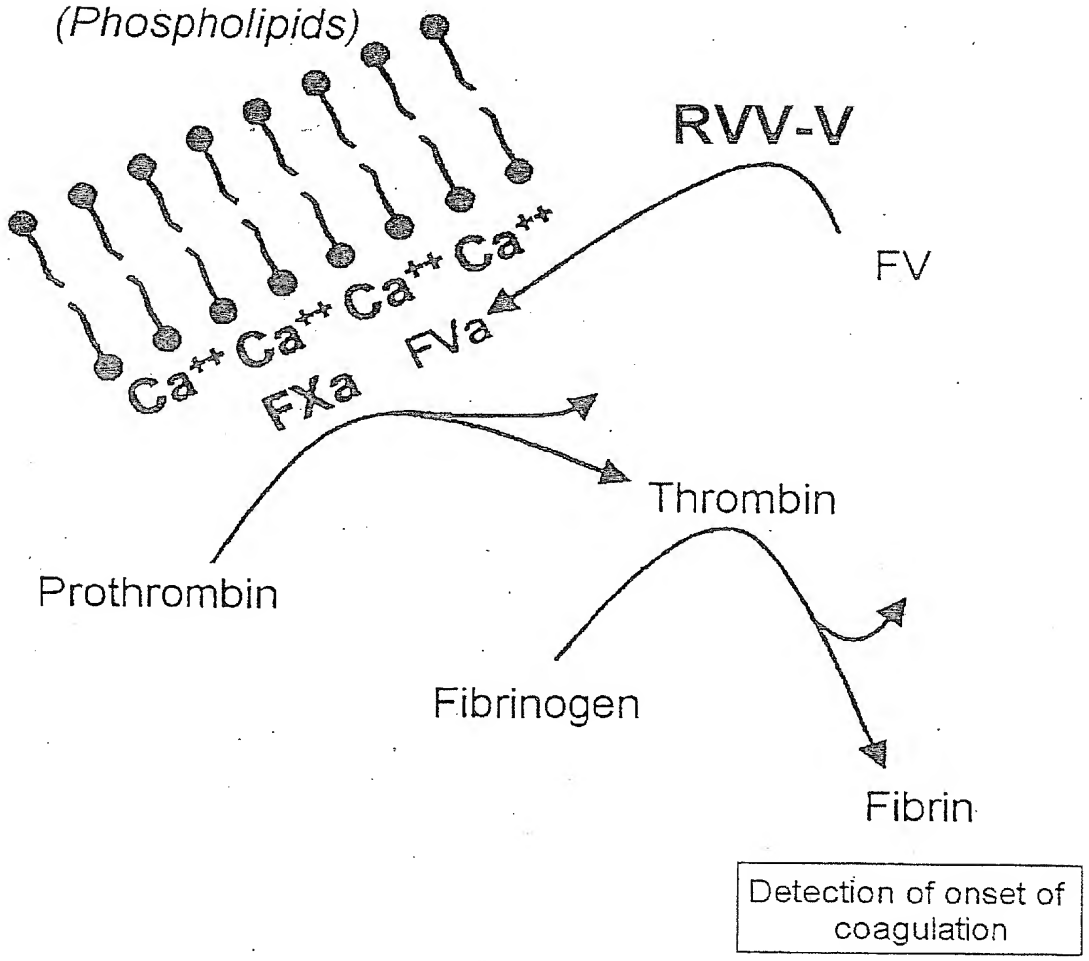


Fig. 5

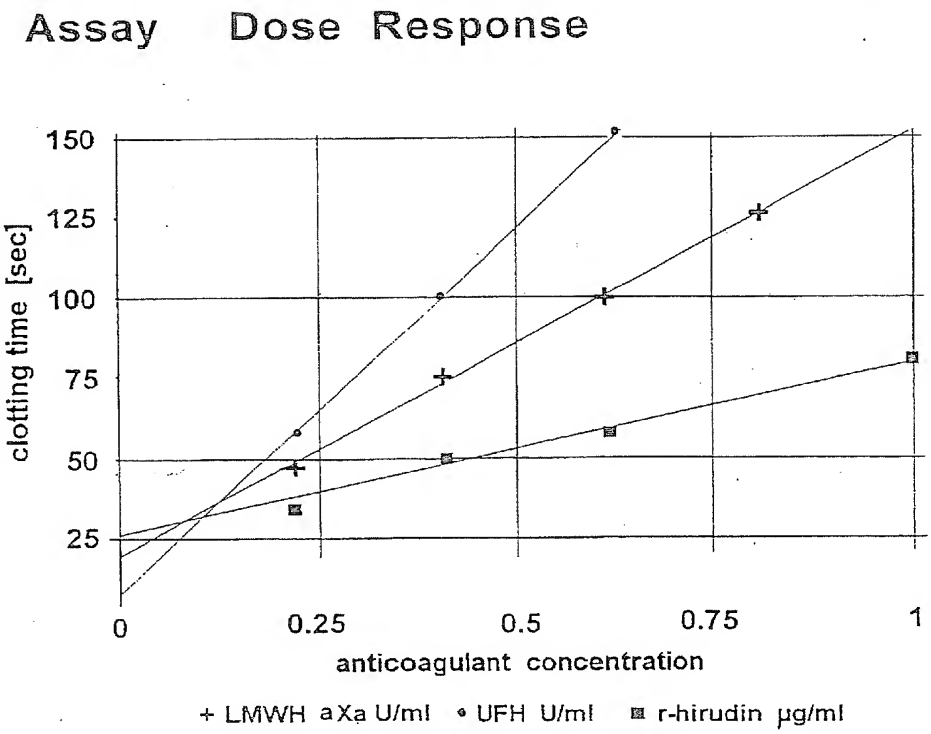
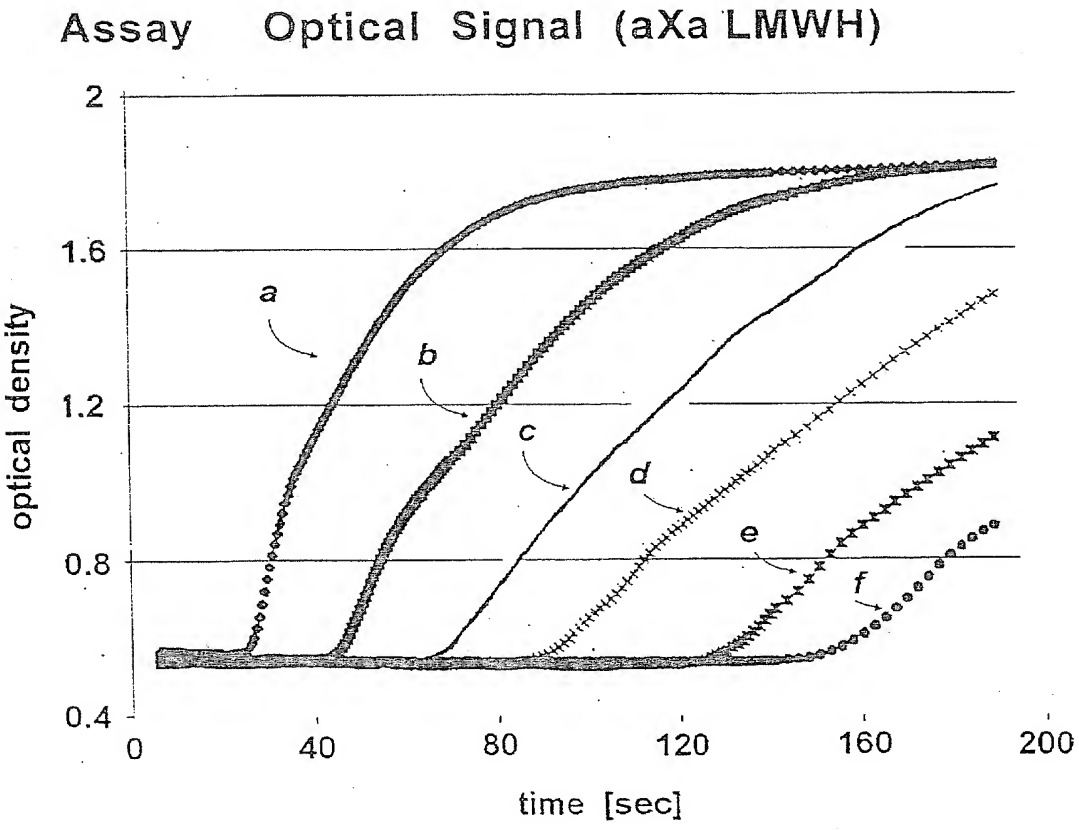



Fig.6




*a control, b 0.2 U/ml, c 0.4 U/ml, d 0.6 U/ml, e 0.8 U/ml, f 1 U/ml*

Fig. 7

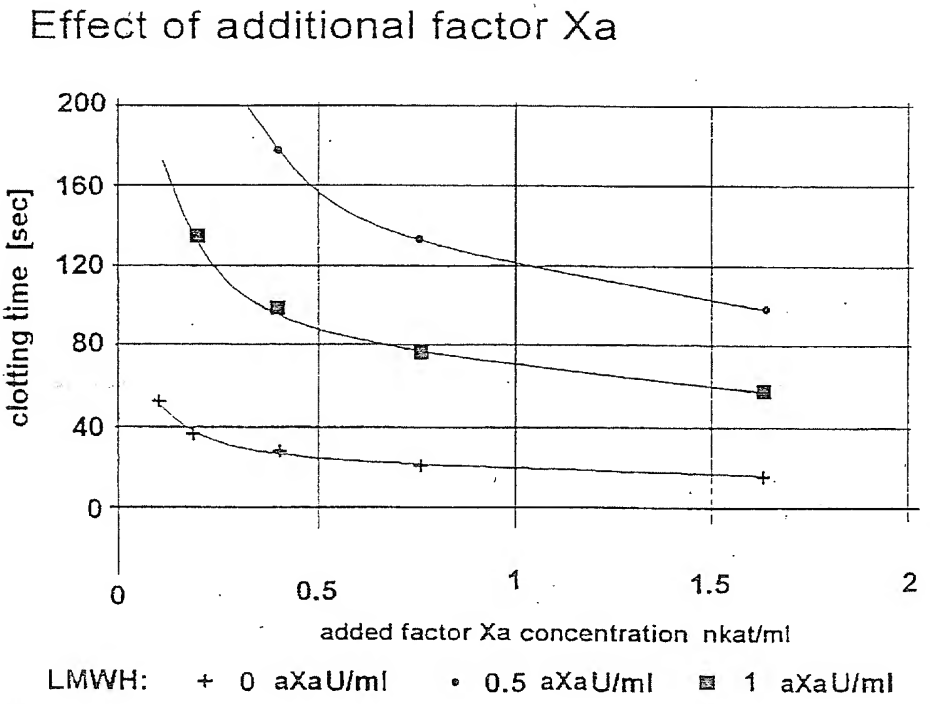



Fig. 8

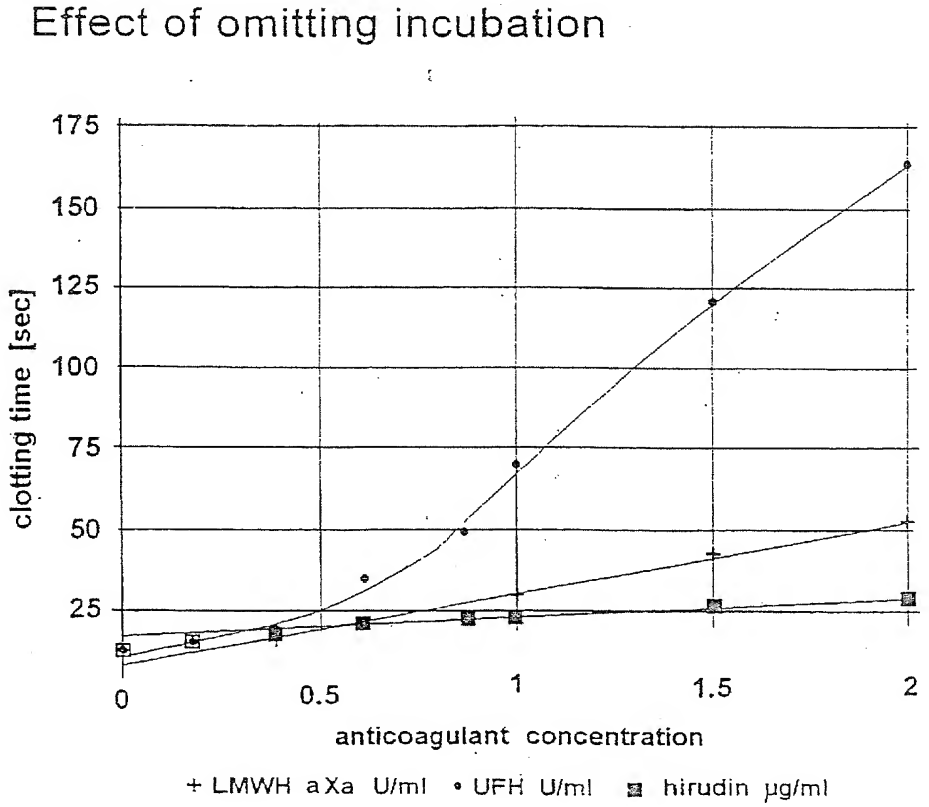
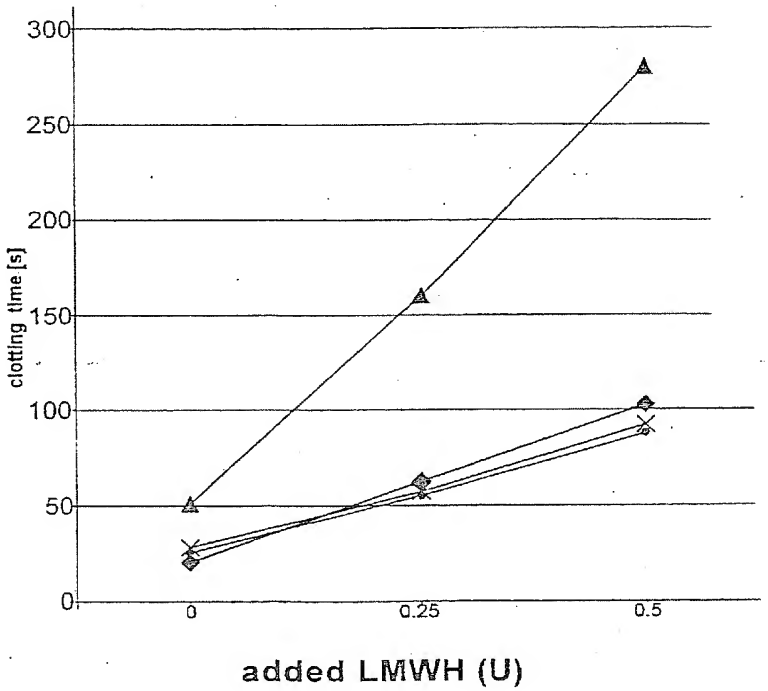


Fig. 9

Patient on oral anticoagulant treatment




◆ Heptest® reference plasma ● Invention reference plasma

▲ Heptest® with anticoagulation ✕ Invention with anticoagulation

Fig 10

Correlation after LMWH Treatment